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Poliovirus mutants with extended (>150-nt) deletions in the 5′-untranslated region between the internal ribosome entry site and the initiator codon have been selected previously (Pilipenko *et al., Cell* 68, 119–131, 1992; Gmyl *et al., J. Virol.* 67, 6309–6316, 1993). These deletions were transferred into the genome of a mouse-pathogenic poliovirus strain and found to be strongly attenuating. The deletions can be considered as covering three structural elements, a stem–loop (domain E) with a conserved cryptic AUG and two spacers, upstream and downstream of it. In an attempt to identify putative essential determinants of neurovirulence in these individual structural elements, appropriate mutants were engineered. The results demonstrated that neither of the above elements is essential for neurovirulence. The results strongly suggested that the presence of a cryptic AUG in the oligopyrimidine/AUG tandem followed, at a sufficient distance, by the initiator codon was necessary to ensure the neurovirulent phenotype of our constructs. On the other hand, the attenuated phenotype appeared to correlate with the occurrence of the initiator AUG as a moiety of the oligopyrimidine/AUG tandem. Possible mechanisms underlying these effects are discussed. Identification of the cryptic AUG as an essential determinant for neurovirulence provides a rational basis for the design of genetically stable attenuated poliovirus variants.

INTRODUCTION

Poliovirus strains vary in the level of their pathogenicity. Upon intracerebral or intraspinal inoculation of monkeys, wild-type strains inflict a severe paralytic, often fatal, disease, whereas attenuated variants (e.g., the Sabin vaccine strains) cause rather mild neurological lesions, usually not accompanied by clinical signs. Extensive studies carried out by several groups demonstrated that mutations in different parts of the poliovirus genome may result in a significant decrease in neurovirulence (for reviews, see Minor, 1992; Agol, 1993). A decade ago, our laboratory put forward a hypothesis that a class of attenuating mutations located in the 5′-untranslated region (5UTR) might specifically interfere with the initiation of translation of the viral RNA in neural cells (Svitkin *et al.,* 1985, 1988). The enhanced ability of the translation machinery of neural cells to "sense" attenuating mutations residing in the 5UTR of the Sabin strains was also supported by experiments with human neuroblastoma cultures (La Monica and Racaniello, 1989; Agol *et al.,* 1989).

Three *cis*-acting RNA elements involved in the translation initiation on picornavirus templates are known to exist. The several-hundred-nucleotide-long internal ribosome entry site (IRES) is responsible for the primary binding of a ribosome (or its smaller subunit) (Pelletier and Sonenberg, 1988; Jang *et al.,* 1988). The IRES-bound ribosome then forms a productive contact with a relatively short downstream template segment called the starting window (Pilipenko *et al.,* 1994). The third element, an appropriately spaced oligopyrimidine/AUG tandem (OAT), also adjoining the IRES from the 3′ side, performs an unidentified function, perhaps stabilizing the ribosome/RNA complex (Beck *et al.,* 1983; Jang and Wimmer, 1990; Nicholson *et al.,* 1991; Pilipenko *et al.,* 1992, 1995; Gmyl *et al.,* 1993). In poliovirus and other enteroviruses, the AUG moiety of the OAT corresponds to a cryptic (noninitiator) AUG located some 150 nucleotides upstream of the initiator codon.

The attenuating mutations that affect the template activity of the RNAs of the Sabin poliovirus strains are located between positions 472 and 481 and thus map to the IRES. Here, we show that alterations in another control element, OAT, may also dramatically change the level of poliovirus neurovirulence, even though their effect on the virus growth in several lines of cultured cells is much milder. This study stemmed from the observation that among a variety of pseudorevertants accumulating after shortening of the spacer between the oligopyrimidine (or box A) and cryptic AUG_{586} moieties of the poliovirus OAT, there was a class with extended deletions encompassing highly conserved elements of the viral RNA 5UTR, including the AUG triplet constituting a part of the wild-type OAT (Pilipenko *et al.,* 1992; Gmyl *et al.,* 1993; see also, Haller and Semler, 1992). Such revertants exhibited a

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reasonably high growth potential *in vitro,* apparently due to the acquisition of a novel OAT composed of the wild-type oligopyrimidine and the initiator AUG_{743}. We attempted to define the functional significance of the portions of the genome lost in these revertants. As shown in this report, the presence of an initiator rather than cryptic AUG within the OAT (and starting window) narrows the host range of the poliovirus by strongly suppressing its *in vivo* growth in the central nervous system, without inflicting comparable changes in the reproductive capacity of the virus or template activity of its RNA *in vitro.* Preliminary data were reported in part at the 8th Meeting of the European Study Group on Molecular Biology of Picornaviruses EUROPIC-94 (Helsinki, 6–11 August 1994) and at the Symposium "RNA Replicon" (Kobe, 12 December 1994).

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli strains HB101, RZ1032, and NM522 were used for propagation of plasmids, for preparation of single-stranded plasmid DNA, and for transformation with mutated double-stranded DNA, respectively.

Plasmids pBM1 (containing nucleotides 68–1131 of Mahoney cDNA cloned into vector pBSM13−) and pBM1/ΔE24P (a pBM1 derivative in which poliovirus 5UTR positions 564–629 were replaced by a synthetic 24-nt-long linker) have been described previously (Pilipenko *et al.,* 1992).

Plasmid pMah(L) (Murray *et al.,* 1988) was a kind gift of Dr. V. Racaniello. It encoded the poliovirus type 1 (virulent Mahoney strain) genome with the replacement of the sequence corresponding to VP1 amino acid residues 95–102 by its counterpart from the mouse-pathogenic type 2 Lansing strain. pMah(L)/Sab was made by replacing the *Kpn*I/*Sca*I fragment (nt 68–987) of Mah(L) by the homologous fragment from pSab1 (kindly donated by Dr. J. Almond), containing the full-length cDNA of Sabin 1.

Construction of mutants

To transfer the extended deletions from PV1/Δ8-74 and -90 (Pilipenko *et al.,* 1992) to Mah(L), the two former viral RNA species were reverse transcribed and their segments between positions 59 and 1078 were subjected to PCR amplification. The amplified cDNA was digested with *Kpn*I and *Sca*I and fragment 68–987 was ligated to pMah(L)-derived fragments *Sca*I/*Bgl*II (poliovirus nucleotides 988–5601) and *Bgl*II/*Kpn*I (the poliovirus genome 5′ and 3′ ends as well as the vector sequences), giving pMah(L)/74 and 90.

All other 5UTR mutations were engineered into plasmids pBM1 or pBM1/ΔE24P. First, a second *Bal*I site was created by site-directed mutagenesis at position 733 (wild-type numbering) of the both plasmids. Then, the plasmids were cut by *Bal*I at positions 630 and 733, and the religation of their large fragments generated pBM1/ΔS and pBM1/ΔES24P, respectively. Insertion, in two orientations, of a 23-nt-long synthetic linker (Pilipenko *et al.,* 1992) into the *Bal*I site of pBM1/ΔES24P gave pBM1/ΔES47P and pBM1/ΔES47M, respectively. pBM1/Δ154, having a 154-nt-long deletion in the 5UTR (nt 579–732), was produced by the procedure involving (i) digestion of unmodified pBM1 with *Bal*I at position 630, (ii) treatment of the linearized plasmid with exonuclease *Bal*31, (iii) its subsequent treatment with the Klenow fragment of DNA polymerase to create blunt ends, (iv) *Kpn*I digestion, and (v) ligation of the poliovirus 5UTR fragment with the large *Kpn*I/*Bal*I fragment of pBM1/ΔS. The above mutations were transferred into pMah(L) by ligation of *Kpn*I/*Sca*I fragments from the mutated pBM1 derivatives as described for pMah(L)/74 and 90.

Transfection and generation of viral stocks

RNA transcripts were synthesized on *Eco*RI-linearized plasmids with phage T7 RNA polymerase and transfected into primary cultures of African green monkey kidney (AGMK) cells by using the DEAE-Dextran technique as described (Pilipenko *et al.,* 1992). Plaques were picked up at Day 4 after transfection, and their material was used to infect RD (human embryonal rhabdomyosarcoma) cell monolayers. Viral stocks were prepared after an additional passage on RD cells. When higher viral titers were required, the viral suspension was clarified by low-speed centrifugation and concentrated to the one-hundredth of the starting volume by using a Millipore Ultrafree-20 filter unit. Plaque titration was carried out on RD or AGMK cells overlaid with a 1% agar medium or on HeLa cells under a semisolid 0.6% agarose medium. The titer of the same viral stock as determined on RD cells was ~100 and ~25 times higher compared to that determined on AGMK and HeLa cells, respectively. The plaques formed on RD cells were significantly larger than those appearing on HeLa cells.

One-step growth curve and *ts* phenotype

The viruses were added to duplicate cultures of HeLa or SK-N-MC (human neuroblastoma; ATCC) cell monolayers at a multiplicity of infection of 20 PFU/cell. After a 60-min incubation at room temperature and rinsing four times with Earle saline, the cells were incubated in Eagle's medium at 36.5 or 39.5°. To determine the time course of viral reproduction, the infected cells were incubated for different periods at the former temperature, whereas a 7-hr incubation at 39.5° served for the *ts* phenotype assay. The incubation was terminated by freezing/thawing, and the virus yield was determined by plaque titration.

In vitro translation

RNA for translation was prepared by successive phenol and phenol–chloroform extractions of the virions pu-

rified by centrifugation in a CsCl gradient. The template activity was assayed in extracts of Krebs-2 cells under the conditions specified previously (Svitkin *et al.,* 1985). The samples (25 μl) were incubated for 60 min at 30° in the presence of 0.5 μg of the respective template RNA. The labeled products were subjected to electrophoresis in SDS–polyacrilamide gels followed by fluorography. The gel regions corresponding to the P1 (capsid proteins precursor) band were excised, and radioactivity was counted in a liquid scintillation spectrometer.

Mouse neurovirulence assay

Male C57Bl mice, 8–12 g, were inoculated intracerebrally with 10-fold dilutions of the viral stock, using six to eight mice for each dilution. Mice were observed daily for 21 days. The virus dose causing paralysis in 50% of mice (PD_{50}) was calculated (Reed and Muench, 1938). The virus was isolated from the spinal cord of at least one mouse paralyzed upon infection with each mutant. After one passage on RD cells, the viral RNA was extracted and checked for the presence of the introduced mutation. Histopathological lesions in the CNS were examined in mice infected with Mah(L). The brains and spinal cords of paralyzed mice were removed and fixed in 10% buffered formalin. Dehydration and embedding in paraffin were achieved through routine histological techniques. The sections of the CNS were prepared, deparaffinized, and stained with gallocyanin.

RESULTS

Neurovirulence of mouse-pathogenic derivatives of poliovirus type 1 Mahoney strain

Since a significant number of the engineered constructs were to be assayed for neurovirulence, it was decided to carry out this study using the mouse as a model animal. Transgenic mice bearing the gene for human poliovirus receptor (Ren *et al.,* 1990; Nomoto *et al.,* 1994) were unavailable to us. Therefore, a derivative of the poliovirus type 1 Mahoney strain capable of causing poliomyelitis in rodents upon intracerebral inoculation (Murray *et al.,* 1988) was used. An octapeptide in the VP1 protein of this derivative was replaced by its type 2 Lansing strain counterpart. The mutant, called here Mah(L), caused the development of flaccid paralysis of fore- and hindlimbs of C57Bl mice in a dose-dependent manner with a PD_{50} value of 1×10^5 PFU (if titration was carried out in monolayers of AGMK cells). This value lied within the range reported in the literature for similar mutants (Murray *et al.,* 1988; Martin *et al.,* 1988; Tardy-Panit *et al.,* 1993). About 95% of the paralyzed mice died.

The validity of the (nontransgenic) mouse model for poliomyelitis has recently been challenged (Gromeier *et al.,* 1995). Therefore, morphological analysis of the central nervous system of the diseased animals was performed. The neuronal pathology in the anterior horns of the spinal cord was typical of poliomyelitis (Fig. 1). This observation confirmed earlier findings (Jubelt *et al.,* 1980). The reason for the disagreement with the observations by Gromeier *et al.* is unknown, but is likely to be due to differences in the mice (cf., Jubelt *et al.,* 1991) and/or virus strains used. In any event, the clinical and pathological signs in mice infected with Mah(L) unambiguously demonstrated that the virus inflicted a poliomyelitis-like disease in these animals.

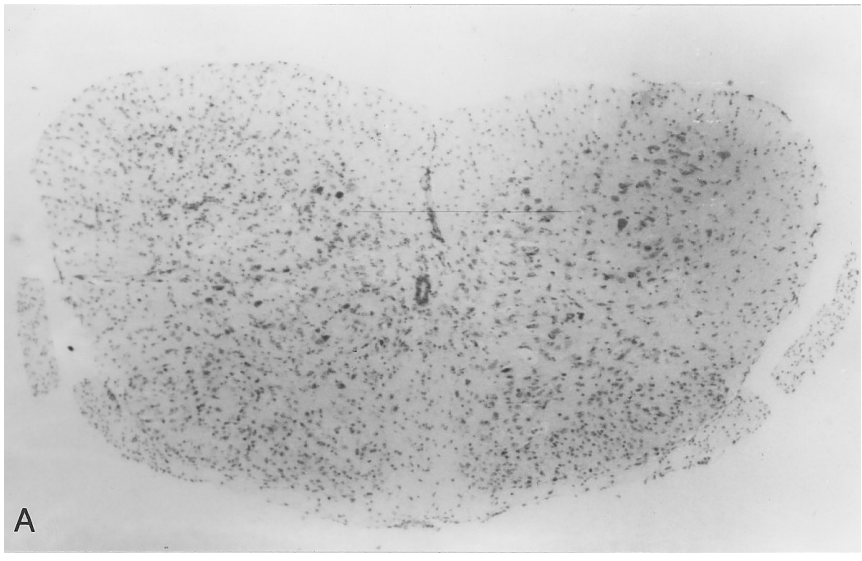
The replacement of positions 68 to 987 in the Mah(L) RNA by the homologous segment from Sabin 1 resulted in a virus, Mah(L)/Sab, having five nucleotide substitutions (four in the 5UTR and one resulting in a $A_{65} \rightarrow S$ change in VP4 protein). PD_{50} of Mah(L)/Sab was 1.3×10^7 PFU, i.e., more than two orders of magnitude higher compared with that of Mah(L). This observation confirmed the feasibility of using mice as a model for the evaluation of attenuating mutations in the 5UTR, as previously proposed (La Monica *et al.,* 1987; see also Tardy-Panit *et al.,* 1993).

Virulence of the mutants with extended deletions

Previously, two mutant viruses, PV1/Δ8-74 and PV1/Δ8-90, with extended deletions expanding from positions 563 to 722 and from positions 562 to 717, respectively, were selected from the progeny of a quasiinfectious RNA having a shortened spacer between the OAT oligopyrimidine and AUG moieties. The mutants grew relatively well in tissue culture cells (AGMK, HeLa, RD) and their RNA exhibited a reasonable template activity in cell-free translation systems derived from Krebs-2 ascites carcinoma cells or rabbit reticulocytes (Pilipenko *et al.,* 1992; Gmyl *et al.,* 1993).

To ascertain the significance of the RNA segments deleted in PV1/Δ8-74 and -90 for viral neurovirulence, the regions encompassing these deletions were reconstructed by cDNA synthesis and PCR amplification and transferred into plasmid pMah(L). The AGMK cells were transfected with respective transcripts, Mah(L)/74 and Mah(L)/90, and the regions corresponding to the replaced segments in the genomes of recovered viruses were sequenced. No fortuitous changes were found (not shown). Both Mah(L)/74 and Mah(L)/90 proved to be highly attenuated for mice, giving PD_{50} values 240 and $>$230 times, respectively, higher than Mah(L) (Table 1). In the case of Mah(L)/90, only 2 mice out of 18 inoculated with the highest available dose (3.6×10^9 PFU; titration on RD cells) developed paralysis. The clinical manifestations of the disease in mice infected with the two viruses with extended deletions also appeared to be milder than those in the case of Mah(L)-inflicted disease: the involvement of forelimbs was never observed, and only about half of the paralyzed mice died during the 21-day observation period.

Thus, the extended deletions in a distal portion of the 5UTR resulted in a significant loss of neurovirulence,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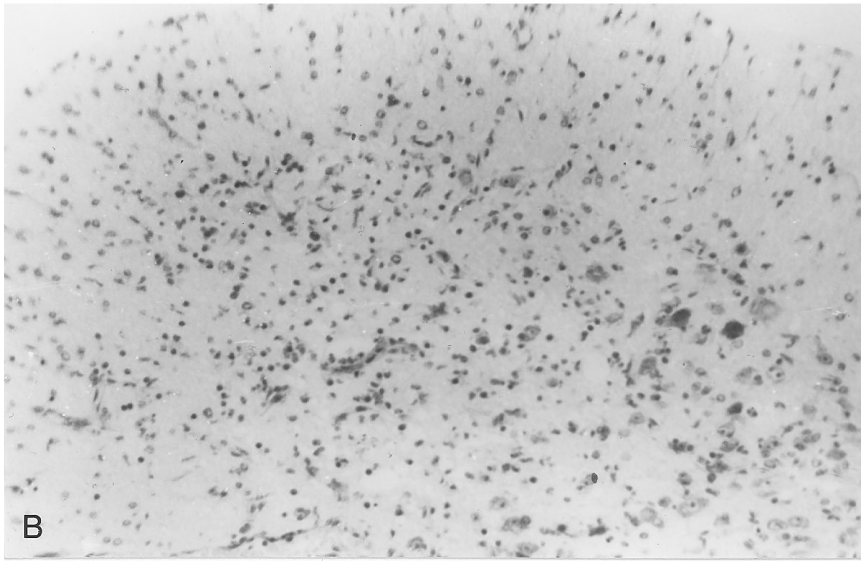
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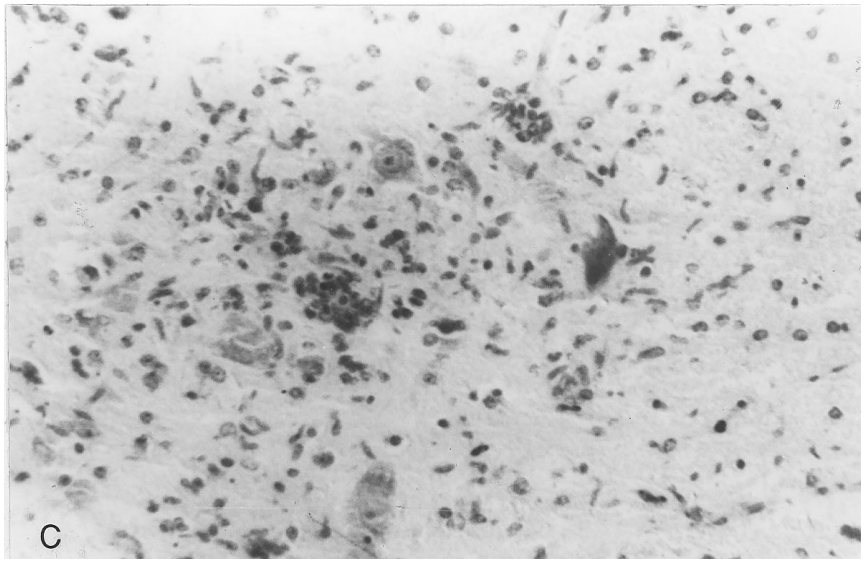
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TABLE 1

Properties of Mutant Viruses

Virus	Genome structure[a] S1	AUG	E	S2	RNA template activity[b]	Plaque size[c] (mm)	*ts* phenotype[d]	Relative attenuation[e]
Mah(L)	+	+	+	+	1.00	4.5	0.20	1
Mah(L)/74	−	−	−	−	0.46	1.6	0.23	240
Mah(L)/90	−	−	−	−	0.18	1.6	0.13	>230
Mah(L)/Δ154	+	−	−	−	0.33	1.6	0.26	107
Mah(L)/ΔE24P	+	+	−	+	0.26	3.1	0.25	1.8
Mah(L)/ΔS	+	+	+	−	1.59	3.4	0.26	1.1
Mah(L)/ΔES24P	+	+	−	−	0.24	3.3	0.16	21
Mah(L)/ΔES47M	+	+	−	−	0.33	3.7	0.14	1.0
Mah(L)/ΔES47P	+	+	−	−	0.34	3.7	0.17	1.7
Sab1	+	+	+	+	nd[f]	nd	<0.0004	nd

[a] The presence (+) or absence (−) of the spacers 1 (S1) and 2 (S2), the cryptic AUG between the IRES and initiator codon, and domain E in the viral genomes are shown.

[b] Template activity of the RNAs is represented by radioactivity of the P1 polyprotein measured according to Materials and Methods and normalized to that of Mah(L).

[c] The average plaque size on RD cells at Day 3.

[d] The ratio of the virus yield (in PFU per HeLa cell) at 39.5° to that at 36.5°.

[e] The ratio of PD_{50} of the mutant to that of Mah(L).

[f] nd, not determined.

suggesting that the deleted segment may contain some determinants specifically required for efficient growth of poliovirus in CNS. The deleted segment can be considered as composed of three structural elements (Fig. 2): (i) a portion of the pyrimidine-rich spacer between domains D and E downstream of the oligopyrimidine moiety of the OAT ($UUUC_{562}$) (spacer 1), (ii) a conserved stem–loop (domain E, nt 581–618) containing the cryptic AUG_{586} component of the wild-type OAT, and (iii) a spacer between domain E and the initiator AUG_{743} (spacer 2). In an attempt to identify putative CNS-specific determinant(s), mutants were engineered in which only one or two of the above elements were absent.

The contribution to neurovirulence of a segment adjoining the oligopyrimidine moiety of the OAT

An aspect in which the OAT in the Mah(L)/74 and Mah(L)/90 RNAs differed from that in the wild-type genome concerned the nucleotide sequence between the oligopyrimidine (box A) element $UUUC_{562}$ and AUG (Figs. 2 and 3). In particular, in the wild-type RNAs there is an additional pyrimidine-rich stretch about 10 nucleotides long (spacer 1) which is not highly conserved (Kuge and Nomoto, 1987; Pöyry *et al.,* 1992; Romero and Rotbart, 1995) but nevertheless is usually present in the genomes of different picornaviruses. To ascertain whether the lack of this stretch was responsible for a lowered virulence of Mah(L)/74 and Mah(L)/90, a mutant was constructed, which was very similar to these viruses but contained a wild-type sequence at positions 563–578. This mutant, Mah(L)/Δ154 (Fig. 3), proved to be highly attenuated, as judged by the PD_{50} value which was found to be only ~2 times lower than in the case of Mah(L)/74 (Table 1); it may be noted, however, that nearly all of the paralyzed Mah(L)/Δ154-infected mice died during the period of observation. Thus, the contribution of the loss of the oligopyrimidine stretch at positions 563–578 to the attenuated phenotype of the mutants with extended deletions was relatively small. The major determinant(s) of neurovirulence should be sought in a downstream locus of the deleted segment.

The significance of conserved domain E

The deleted segment included a highly conserved stem–loop, domain E. This domain contained, along with other elements, the cryptic (noninitiator) AUG component of the wild-type poliovirus OAT. The straightforward deletion of this domain resulted in a dead construct, apparently as a result of the removal of the AUG moiety of the OAT. However, the replacement of the domain by a short synthetic AUG-containing linker generated a viable large-plaque-former PV1/ΔE24P (Pilipenko *et al.,* 1992). When

FIG. 1. Histopathological lesions in the spinal cord of mice infected with Mah(L). Gallocyanin-stained sections of the cervical cord from a mouse with a paralyzed left forelimb 2 days after onset of the disease. (A) Nearly complete loss of motor neurons in the left ventral horn, while the right ventral horn showed only mild alterations (original magnification, ×32). (B) A higher magnification of a part of the left ventral horn from (A), showing loss of motor neurons, disseminated glial reaction, and vascular infiltrations (original magnification, ×65). (C) A section showing various degrees of neuron destruction and neuronophagia in a ventral horn (original magnification, ×200).

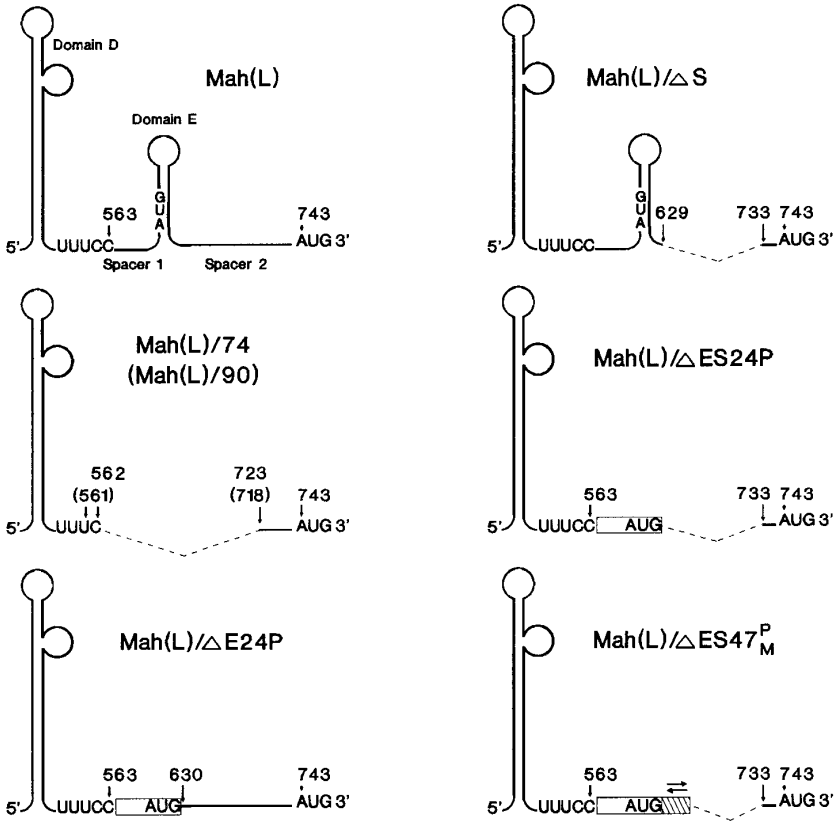

FIG. 2. Schematic representation of a distal portion of the 5UTR of wild-type poliovirus [Mah(L)] and engineered mutants. The open and the hatched bars correspond to synthetic 24-nt-long AUG-containing and 23-nt-long linkers, respectively. Arrows above the hatched bar indicate orientation of the 23-nt-long linker in Mah(L)/ΔES47P and Mah(L)/ΔES47M.

the altered RNA segment of the latter mutant was transferred into the Mah(L) background, the resulting Mah(L)/ΔE24P (Figs. 2 and 3) turned out to be as neurovirulent as its parent Mah(L), judging by both the PD_{50} value (Table 1) and clinical signs.

Thus, the attenuated phenotype of the mutants with extended deletions could not be explained simply by the lack of the secondary structure domain E.

The significance of the spacer between domain E and the initiator AUG

The spacer between domain E and the initiator AUG (spacer 2, Fig. 2), being variable with respect to the nucleotide sequence (Kuge and Nomoto, 1987; Pöyry *et al.,* 1992), has a constant length of 126–130 nt in enterovirus genomes. Its physiological significance remains unknown. To ascertain whether it contains a determinant ensuring efficient poliovirus growth in the CNS, a mutant lacking specifically this spacer, Mah(L)/ΔS (Figs. 2 and 3), was engineered and assayed for neurovirulence. This mutant retained full pathogenicity of its parent Mah(L) (Table 1), suggesting that spacer 2 contained no element essential for efficient poliovirus reproduction *in vitro* or *in vivo.*

Neurovirulence of mutants lacking both domain E and the downstream spacer

Thus, the above experiments failed to demonstrate the existence of a unique neurospecific determinant within the segment absent from the Mah(L)/74 or Mah(L)/90 RNAs. Taking into account that positions 670–772 in the poliovirus type 1 RNA represent an imperfect repeat of positions 533–645 (Pilipenko *et al.,* 1990), the possibility still existed that the lost putative determinant might be present in two copies, either of which being sufficient to enable the virus to efficiently grow in the CNS. Therefore, mutants were designed in which both domain E and the downstream spacer were deleted. Three such constructs, Mah(L)/ΔES24P, Mah(L)/ΔES47P, and Mah(L)/ΔES47M, were engineered (Figs. 2 and 3). All of them had an identical synthetic oligonucleotide containing a cryptic AUG at a proper distance (20 nt) from the oligopyrimidine moiety of the OAT as well as 7 parental nucleotides preceding the initiator AUG_{743}. In addition, mutants Mah(L)/ΔES47P and Mah(L)/ΔES47M contained a synthetic 23-nt-long linker between the cryptic and initiator AUGs in the two opposite orientations, respectively. These latter mutants exhibited full virulence of their parent, Mah(L) (Table 1), indicating that all the elements required for the pathogenic potential of the virus were present in these constructs.

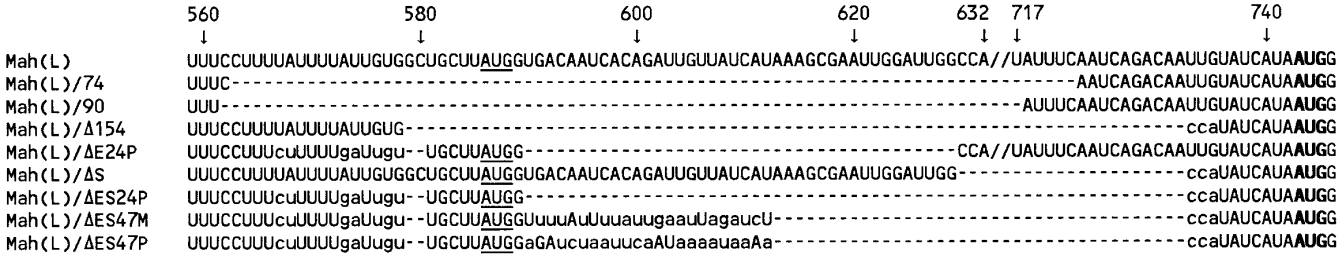

FIG. 3. The alignment of the mutant 5UTR segments. The nucleotides that differ from those in Mah(L) are in lowercase letters. Gaps were introduced manually to maximize similarity. The cryptic and initiator AUGs are underlined and printed in boldface, respectively. Nucleotides corresponding to positions 633–716 in Mah(L) and Mah(L)/ΔE24P are not shown for the sake of brevity.

On the other hand, Mah(L)/ΔES24P (Figs. 2 and 3), which lacks the synthetic linker between the cryptic and initiator AUGs, exhibited a moderately (~20-fold) decreased level of virulence (Table 1). This fact suggested that the distance between different genomic elements might contribute to the poliovirus ability to grow in the CNS.

Some *in vitro* properties of the mutants

The following *in vitro* properties of the mutants were investigated: (i) the one-step growth curve in HeLa and human neuroblastoma SK-N-MC cells, (ii) the plaque phenotype, and (iii) the *ts* phenotype. Figure 4 demonstrates that the final yields, in terms of PFU/cell, of all of the attenuated and virulent mutants were essentially similar when tested in a given culture, differing not more than threefold from that of Mah(L). It may be noted that the harvest of Sabin 1 strain in SK-N-MC cells was two orders of magnitude lower compared to that of Mah(L) (1.7 vs 270 PFU/cell; not shown), confirming our previous results (Agol *et al.,* 1989). In both cultures, however, the latent period was 1–2 hr longer in the case of highly attenuated variants (Mah(L)/74, /90, and /Δ154) compared to that of the virulent strains. This observation was consistent with the results reported by Iizuka *et al.* (1989). The latent period of the virus with an intermediate level of virulence, Mah(L)/ΔES24P, was somewhat prolonged in HeLa but not SK-N-MC cells.

The highly attenuated mutants, compared to Mah(L), produced markedly smaller plaques on RD cells (Table 1), whereas the difference was not so pronounced on HeLa monolayers (1.5–2.0 mm vs 2–3 mm; not shown). None of the mutants exhibited a *ts* phenotype (Table 1).

Thus, only a limited correlation between the neurovirulence and reproductive capacity in cultured cells (even those of neural origin) could be registered.

The *in vitro* template activity of the mutant RNAs

The template activity of the genomic RNA from the mutants was assayed in mRNA-dependent Krebs-2 extracts. No correlation between the level of neurovirulence and the template activity was found. Most of the mutant RNA species exhibited a lowered protein-synthesizing activity, but this deficiency was nearly equally expressed in the attenuated (18–46% of the control in the case of Mah(L)/74, /90/, and /Δ154) and all but one virulent (26–34%) strains (Fig. 5, Table 1). An exceptionally high activity (150% of the control) was exhibited by the Mah(L)/ΔS RNA, lacking spacer 2.

DISCUSSION

The region of the poliovirus downstream of the IRES has a peculiar organization. The IRES and the initiator codon are separated by ~150 nucleotides. This region contains a highly conserved stem–loop (domain E) and two spacers, lying upstream and downstream of the stem–loop, respectively (Fig. 2). The spacers are conserved with respect to the length rather than to the primary structure. The physiological significance of these structural elements remains obscure. Domain E contains a conserved cryptic (i.e., in a poor context) AUG, which plays a role in the translation initiation (Pelletier *et al.,* 1988). This AUG is a component of the OAT (oligopyrimidine/AUG tandem), a *cis*-element that is required for efficient translation initiation in a host-dependent and IRES-dependent manner (Pilipenko *et al.,* 1995). The distance between the oligopyrimidine (box A) and AUG moieties of the OAT should lie within a certain range, and this rule may be responsible for the maintenance of a constant length of spacer 1. In addition, the AUG moiety of the OAT maps to the starting window, the position of which is also determined by a constant distance from the IRES (Pilipenko *et al.,* 1994). On the other hand, we are aware of no reliable explanations for the conservation of either the secondary structure of domain E or the constant length of spacer 2.

The physiological significance of the two latter structural elements might be questioned by the discovery that they could be deleted (without a dramatic loss of the ability to grow in tissue culture cells) either by genetic engineering (Kuge and Nomoto, 1987; Iizuka *et al.,* 1989) or as a natural response to other mutations (Pilipenko *et al.,* 1992; Gmyl *et al.,* 1993; Haller and Semler, 1992). The OAT destroyed in such deletion mutants by the elimination of the cryptic AUG was recreated due to the placement of the initiator AUG at the proper distance from the oligopyrimidine.

The combined loss of domain E and the downstream

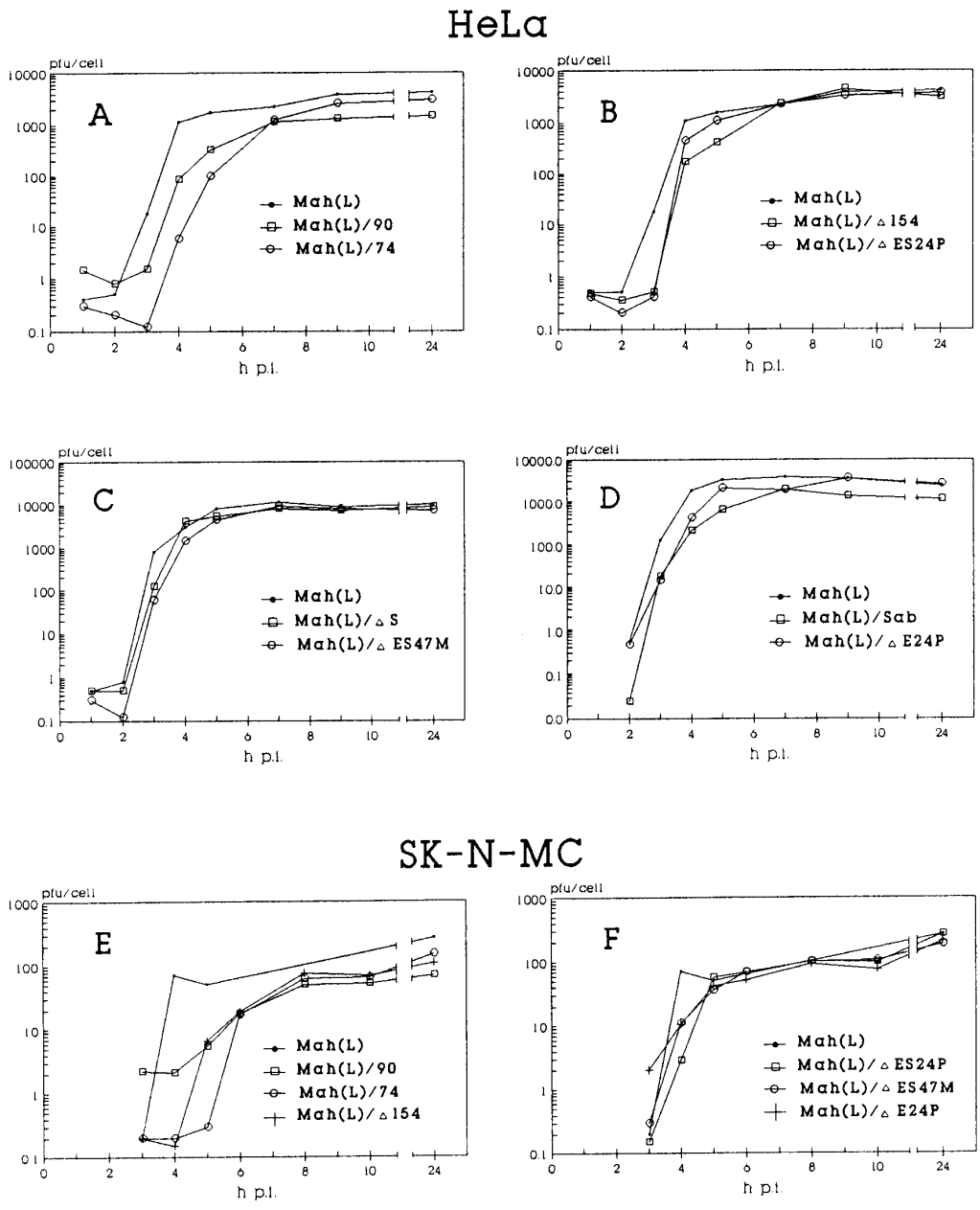

FIG. 4. The time course of viral reproduction in HeLa and SK-N-MC cells at 36.5°.

spacer resulted, however, in a significant loss of neurovirulence as shown here and previously (Iizuka *et al.,* 1989). This fact raised the possibility that the deleted region might contain some element(s) specifically needed for efficient poliovirus reproduction in the CNS. A search for such an element carried out in this study clearly demonstrated, however, that the mutants lacking domain E or spacer 2 or both could be as virulent as their wild-type parent. This was the case, for example, with the engineered mutants Mah(L)/ΔES47P and Mah(L)/ΔES47M. There is, however, an important genomic difference between these virulent mutants and the attenuated ones, Mah(L)/74 and Mah(L)/90. The two former viruses have a cryptic (poor context) AUG at a proper distance from the IRES, whereas the latter two have the initiator AUG. This correlation between the neurovirulence and the presence of a cryptic AUG between the IRES and the initiator (good context) codon is characteristic of all the engineered mutants studied here but Mah(L)/ΔES24P. This exception from the rule will be discussed below.

On the basis of the above findings, we propose that efficient translation of poliovirus RNA in the CNS demands that the OAT (or starting window) should contain a cryptic rather than the initiator AUG, a situation not considered in the original formulation of the starting window hypothesis. The contact of the IRES-bound ribosome directly with the initiator codon will probably result, in this particular host system, in the formation of an abortive, rather than productive, complex. In other words, the initiator codon-containing OAT may, in this case, stabilize the contact of the IRES-

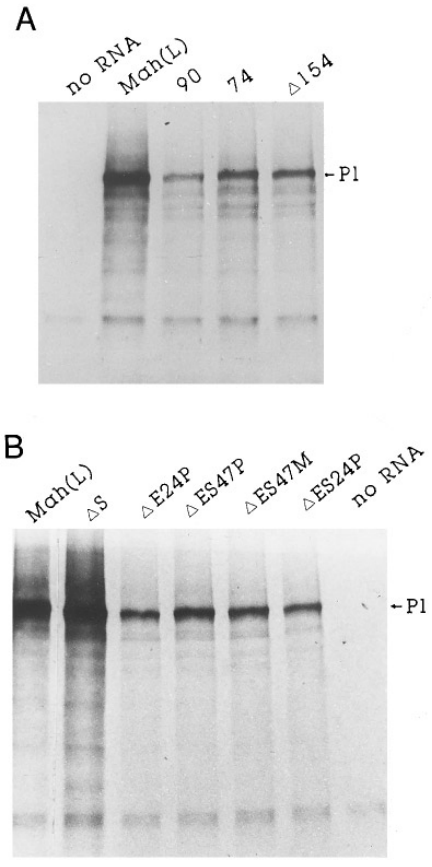

FIG. 5. Translation of the viral RNAs in extracts of Krebs-2 cells. The reactions were programmed with the mutant RNAs lacking (A) and possessing (B) a cryptic AUG between the IRES and the initiation codon. The position of the P1 (capsid protein precursor) band is indicated.

bound ribosome with the starting window either inefficiently or, on the contrary, too strongly; in both cases further movement of the ribosome along the template will be impeded. Partial attenuation of Mah(L)/ΔES24P can then be explained by the proximity of the cryptic and initiator AUGs to each other. If they are both within the starting window, some of the IRES-bound ribosomes may form abortive contacts with the initiator codon. An increase in the distance between the two AUGs, as in Mah(L)/ΔES47P and Mah(L)/ΔES47M, should take away the initiator AUG from the starting window, thereby preventing the abortive interaction. Such a preference for a cryptic AUG in the OAT appears to be much weaker, if exists at all, in the cultured cells studied here.

Obviously, the difference between cryptic and initiator AUGs lies in their respective contexts. Therefore, the discriminating tools, whatever their nature, should recognize not merely the AUG triplets but their context as well.

The molecular basis for the proposed host-dependent difference in the specificity of translation machinery is unknown but the phenomenon itself is not without precedents. Recently, our laboratory reported that neurovirulence of Theiler's murine encephalomyelitis virus (TMEV) depends on the presence of the OAT, whereas this element is not important for the reproduction of the virus in at least certain lines of cultured cells (Pilipenko *et al.,* 1995). Remarkably, in the case of TMEV, the insertion of a cryptic AUG between the IRES and the initiator codon (which in this virus is a moiety of the OAT) was accompanied by a loss of neurovirulence, an effect quite the opposite to that observed with poliovirus.

Among the poliovirus 5UTR translational *cis*-elements, the OAT is not the only entity affecting the efficiency of viral growth in the CNS in a host-dependent fashion. Point mutations within the IRES (in particular, within the region with coordinates 472–481) contribute to the attenuated phenotype of the Sabin strains (Minor, 1992) and impair the *in vitro* template activity of the viral RNA (Svitkin *et al.,* 1985, 1988, 1990). Destabilization of the secondary structure of the respective domain also results in a decrease in virulence and in translational defects (Skinner *et al.,* 1989; Haller *et al.,* 1996). The neurospecificity of these mutations appears to be due to peculiarities of the set of translation initiation factors in the neural cells (Haller *et al.,* 1996). The specific factor requirements may differ in the cases of the attenuating mutations mapping to either the IRES or the OAT. Thus, as shown in the present study, the latter, in distinction with the former, may not be accompanied by the translation deficiency of the viral RNA in Krebs-2 extracts or inefficient growth in human neuroblastoma cells.

It was originally proposed by Nomoto *et al.* (1988) to consider poliovirus mutants with extended deletions in the 5UTR as candidates for live poliovirus vaccine. Now we begin to understand the theoretical basis underlying this proposal. The mutants described here and by the Nomoto's group (Iizuka *et al.,* 1989) are not only highly attenuated but also genetically stable. Their reversion to neurovirulence, if possible at all, should involve complicated genetic rearrangements rather than simple point mutations characteristic of reversions in the Sabin strains. Obviously, the use of such engineered mutants as vaccine candidates demands that they should grow efficiently in nonneural tissues of vaccinees. This property of the mutants remains to be tested.

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